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In the Virion, the 11-Amino-Acid Peptide Cofactor pVIc Is Covalently Linked to the Adenovirus Proteinase

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Previously, the adenovirus proteinase (AVP) had been shown to be stimulated by an 11-amino-acid cofactor pVIc; the crystal structure of an AVP–pVIc complex formed *in vitro* reveals a disulfide bond between AVP and pVIc. However, that disulfide bond was recently shown not to be required for maximal stimulation of enzyme activity by pVIc *in vitro.* Is the disulfide bond physiologically relevant or is it an artifact that arose in the crystallization of the complex? Here we show that a disulfide bond between AVP and pVIc is physiologically relevant, because in the virus particle AVP is linked to pVIc via a disulfide bond. This is also the first experimental proof that AVP interacts *in vivo* with one of its cofactors, all of which were discovered and characterized *in vitro.* A rationale as to why this apparently unnecessary disulfide bond between AVP and pVIc forms in the virus particle is presented.

INTRODUCTION

The human adenovirus serotype 2 proteinase (AVP) is required for the synthesis of infectious virus (Weber, 1976). Late in infection, young virions are formed in which 6 of the 12 major virion proteins are precursor proteins. In the young virion, about 70 AVP molecules become activated (Brown *et al.,* 1996), and they carry out over 3200 cleavages of multiple copies of the 6 precursor proteins, thereby rendering a virus particle infectious (Rancourt *et al.,* 1995). A temperature-sensitive mutant of adenovirus was shown to lack proteinase activity at the nonpermissive temperature (Weber, 1976). The mutation was mapped to the L3 23K gene (Yeh-Kai *et al.,* 1983). The L3 23K gene was cloned and expressed in *Escherichia coli* (Anderson, 1993; Mangel *et al.,* 1993) or baculovirus-infected insect cells (Webster *et al.,* 1993) and the resultant 204-amino-acid protein AVP was purified (Mangel *et al.,* 1993; Webster *et al.,* 1993).

In vitro, the recombinant form of AVP is relatively inactive (Baniecki *et al.,* 2001; Mangel *et al.,* 1993; McGrath *et al.,* 2001a; Webster *et al.,* 1993). Eventually, cofactors were discovered. One cofactor is the 11-amino-acid peptide, GVQSLKRRRCF, originating from the C-terminus of the precursor to protein VI, pVIc. The other cofactor is the viral DNA (Mangel *et al.,* 1993). *In vitro,* the two viral cofactors increase the specificity constant, k_{cat}/K_m, for substrate hydrolysis (McGrath *et al.,* 2001a). If the relative k_{cat}/K_m for AVP alone is 1, for an AVP–pVIc complex it is 1130, for an AVP–DNA complex it is 110, and for an AVP–pVIc–DNA complex it is 34,100.

The crystal structure of the AVP–pVIc complex reveals where and how pVIc binds to AVP (Ding *et al.,* 1996). Cys104 of AVP forms a disulfide bond with Cys10′ of pVIc; both Cys104 and Cys10′ are conserved among adenovirus serotypes. The active-site nucleophile, the Cys122–His54 ion pair, is located in a groove on the surface of the enzyme. Surprisingly, pVIc, which greatly increases the k_{cat}/K_m for substrate hydrolysis by AVP, binds quite far from the active-site nucleophile; the pVIc Cys10′ residue is 32 Å away from Cys122. The interaction between pVIc and AVP is extensive. There are 24 non-β-strand hydrogen bonds and 6 β-strand hydrogen bonds, as well as the covalent bond.

The disulfide bond between pVIc and AVP is not required for maximal stimulation of AVP by pVIc (McGrath *et al.,* 2001b). At a concentration of pVIc fivefold greater than its equilibrium dissociation constant, K_d, for AVP, the half-time for formation of a disulfide bond with AVP is 29 min. Yet, when the same concentration of pVIc is added to a solution of AVP and substrate, there is a lag of only 3 min before the maximal rate of substrate hydrolysis is reached. Because upon addition of pVIc to AVP and substrate, the maximal rate of substrate hydrolysis is reached before a disulfide bond can form, formation of the disulfide bond is therefore not necessary for maximal stimulation of AVP by pVIc. This then raises the question as to whether the disulfide bond observed in the crystal structure of AVP–pVIc (Ding *et al.,* 1996) is physiologically relevant or is an artifact that arose by the high concentrations of AVP and pVIc used in the formation and crystallization of the complex.

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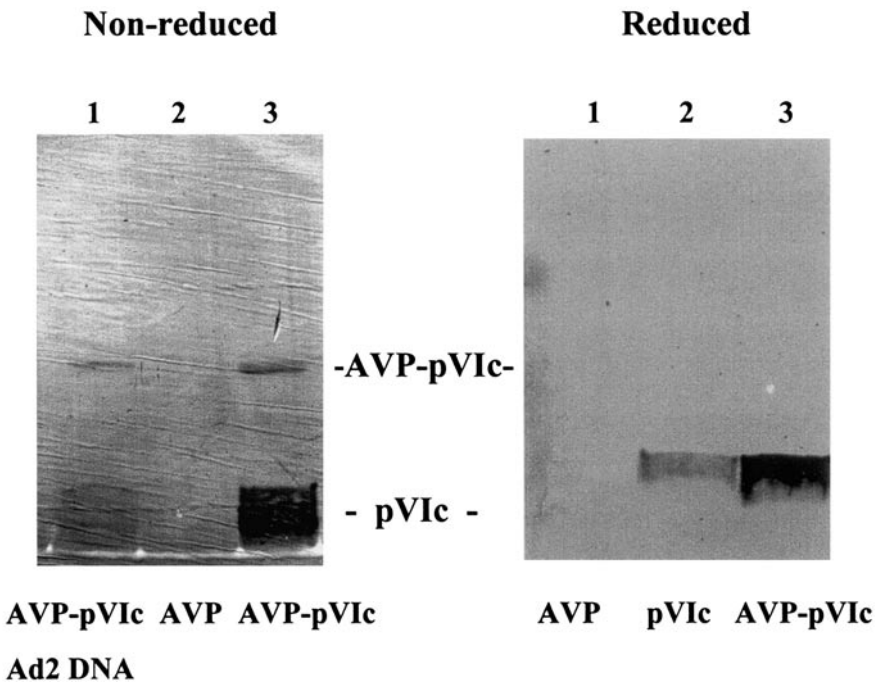

FIG. 1. Demonstration of the formation of a disulfide bond formed *in vitro* between pVIc and AVP, in the presence or absence of Ad2 DNA, as assayed by immunoblotting. Samples for the nonreduced gel contained in lane 1 (0.015 μg AVP–pVIc complexes formed by incubating overnight 2 μM AVP with 2.5 μM pVIc and 17 μg/ml Ad2 DNA, as described under Materials and Methods), in lane 2 (0.03 μg AVP), and in lane 3 (0.03 μg AVP–pVIc complexes formed by incubating overnight 7 μM AVP with 50 μM pVIc, as described under Materials and Methods). Samples for the reduced gel contained in lane 1 (0.03 μg AVP), lane 2 (0.03 μg pVIc), and lane 3 (0.03 μg AVP–pVIc complexes formed by incubating overnight 7 μM AVP and 50 μM pVIc, as described under Materials and Methods). After SDS–PAGE, the proteins in the gels were transferred to BSA-containing Immobilon membranes, fixed with glutaraldehyde, and immunoblotted with anti-pVIc antibody.

If the disulfide bond is physiologically relevant, then there should be covalently linked AVP–pVIc complexes in the virus particle. To determine directly whether a disulfide bond forms between AVP and pVIc *in vivo,* in the virus particle, a series of Western blots was run under reducing and nonreducing conditions using a new method to enhance retention of small peptides on membranes during immunoblotting. The data indicated that in the virus particle, there is an AVP–pVIc complex linked by a disulfide bond. This conclusion is also the first evidence that AVP interacts with a cofactor *in vivo;* heretofore, all the experiments with AVP cofactors were done *in vitro.* Finally, we present a rationale for the involvement of disulfide bond formation in the activation of AVP in the virus particle, given that the bond is not necessary for maximal stimulation of enzyme activity by pVIc.

RESULTS

Demonstration that pVIc forms a disulfide bond with AVP *in vitro*

Formation of a disulfide bond between AVP and pVIc *in vitro* in the presence or absence of Ad2 DNA was shown by a direct method, Western blotting (Towbin *et al.,* 1979), using a new protocol designed to increase retention of peptides on membranes. AVP–pVIc complexes were formed in the presence of 17 μg/ml Ad2 DNA by incubating 2 μM AVP with 2.5 μM pVIc, a concentration of pVIc 28-fold higher than its K_d for AVP in the presence of DNA (Baniecki *et al.,* 2001). AVP–pVIc complexes were formed in the absence of Ad2 DNA by incubating 7 μM AVP with 50 μM pVIc, a concentration of pVIc 12-fold higher than its K_d in the absence of DNA. Then, both nonreduced and reduced SDS–polyacrylamide gels were run. For the nonreduced gel, β-mercaptoethanol was omitted from the SDS–PAGE sample buffer. After SDS–PAGE, the proteins in the gels were transferred to bovine serum albumin (BSA)-treated membranes, fixed with glutaraldehyde, and immunoblotted using affinity-purified polyclonal antibodies raised against pVIc. pVIc, which has two primary amines, should cross-link to BSA.

The data are shown in the nonreduced and reduced Western blots in Fig. 1. In the nonreduced blot, a high-molecular-weight complex with pVIc is observed in lane 1 where AVP was incubated with pVIc and DNA and in lane 3 where AVP was incubated with pVIc. That these high-molecular-weight complexes are AVP–pVIc complexes held together by disulfide bonds is shown by the experiment in the reduced blot. In lane 3 of the reduced blot, the AVP–pVIc complex that migrated as a high-molecular-weight complex in lane 3 in the nonreduced

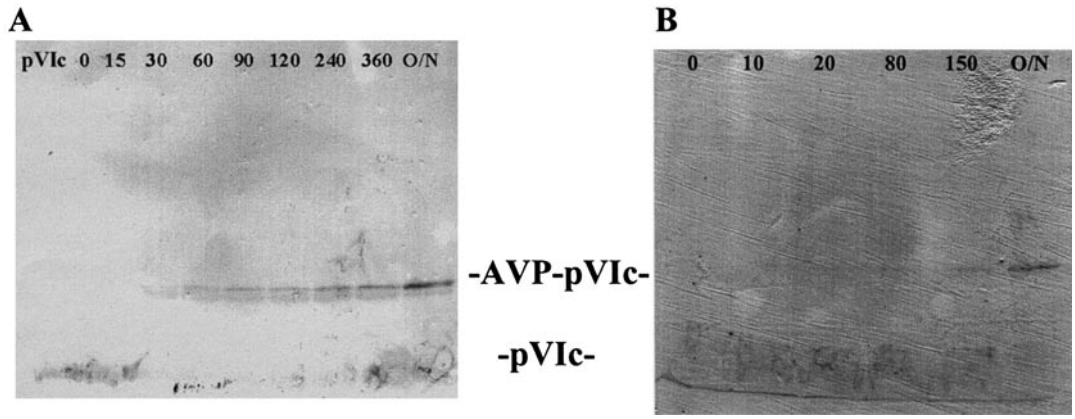

FIG. 2. Kinetics of disulfide bond formation *in vitro* between pVIc and AVP in the absence (A) or in the presence (B) of Ad2 DNA, as assayed by immunoblotting. (A) Reactions in the absence of DNA contained 7 μM AVP and 50 μM pVIc and were incubated for the indicated periods of time after which aliquots were diluted 15-fold for nonreducing SDS–PAGE. (B) Reactions in the presence of DNA contained 17 μg Ad2 DNA, 2 μM AVP, and 2.5 μM pVIc and were incubated for the indicated periods of time after which aliquots were diluted 15-fold for nonreducing SDS–PAGE. After SDS–PAGE, the proteins in the gels were transferred to BSA-treated Immobilon membranes, fixed with glutaraldehyde, and immunoblotted with anti-pVIc antibody. pVIc signifies pVIc alone. The times, 0–360, are in minutes. O/N signifies overnight.

blot now migrates as pVIc. pVIc alone is in lane 2 in the reduced blot.

The blots showed that the anti-pVIc antibody was specific for pVIc. The data in lane 2 of the nonreduced blot and in lane 1 of the reduced blot showed that the anti-pVIc antibody did not react with AVP. In lanes 2 and 3 of the reduced blot the anti-pVIc antibody reacted with a band that migrated in a position where pVIc should be, i.e., near the bottom of the membrane. In the nonreduced blot, the band of pVIc is darker in lane 3 than in lane 1. This is because lane 3 has sixfold more pVIc than lane 1. These data clearly show that the method can be used to detect a disulfide bond formed *in vitro* between pVIc and AVP and that a disulfide bond was able to form *in vitro* between pVIc and AVP in the presence or absence of Ad2 DNA.

Kinetics of formation of a disulfide formed *in vitro* between pVIc and AVP

The rate of formation of a disulfide bond *in vitro* is relatively slow, much slower than the rate of formation of a reversible AVP–pVIc complex (McGrath *et al.,* 2001b). AVP was incubated with pVIc in the absence or presence of Ad2 DNA and, after varying time intervals, aliquots of the reactions were diluted 15-fold in nonreducing SDS–PAGE buffer. After SDS–PAGE, the proteins in the gels were transferred to BSA-treated Immobilon membranes, fixed with glutaraldehyde, and immunoblotted with anti-pVIc antibody. The results (Fig. 2A in the absence of Ad2 DNA and Fig. 2B in the presence of Ad2 DNA) showed that the amount of disulfide-bonded complex increased as a function of time. Very little AVP had formed a disulfide bond with pVIc within 1 h; the majority of disulfide-bonded complex required more than 6 h to form, in the absence or presence of Ad2 DNA. In this method, any pVIc that migrates with AVP is considered to have formed a disulfide bond with AVP.

Demonstration that *in vivo,* in the virus particle, pVIc formed a disulfide bond with AVP

If a disulfide bond between pVIc and AVP is not necessary for maximal stimulation of enzyme activity (McGrath *et al.,* 2001b), then does a disulfide bond form *in vivo* in the virus particle? To answer this question, disrupted wild-type Ad2 virions, prepared by incubation at 56°C for 20 min in 10 mM HEPES (pH 8.0) and 5 mM iodoacetamide, were placed in SDS–PAGE sample buffer in the absence or presence of 5% β-mercaptoethanol and then sonicated for 5 min before being heated in a boiling water bath for 3 min. After SDS–PAGE, the proteins in the gel were transferred to BSA-treated membranes, cross-linked by glutaraldehyde, and immunoblotted. The data are shown in Fig. 3. In lane 1, the nonreduced gel, there are bands immunoreactive to anti-pVIc antibody at the AVP–pVIc position and at the pVIc position. In lane 2, the reduced gel, there is a band immunoreactive to anti-pVIc antibody only at the pVIc position.

A control experiment was performed to confirm that the immunoreactive band at the AVP–pVIc position in lane 1 of the nonreduced gel in Fig. 3 that reacted with anti-pVIc antibody is indeed an AVP–pVIc complex in the virus. This is shown in the reduced blot in Fig. 3, lane 3, where the band illuminated by anti-AVP antibody migrated to the same position as the band in lane 1 that was illuminated by anti-pVIc antibody. These data clearly showed that pVIc had formed a disulfide bond with AVP *in vivo,* in the virus particle.

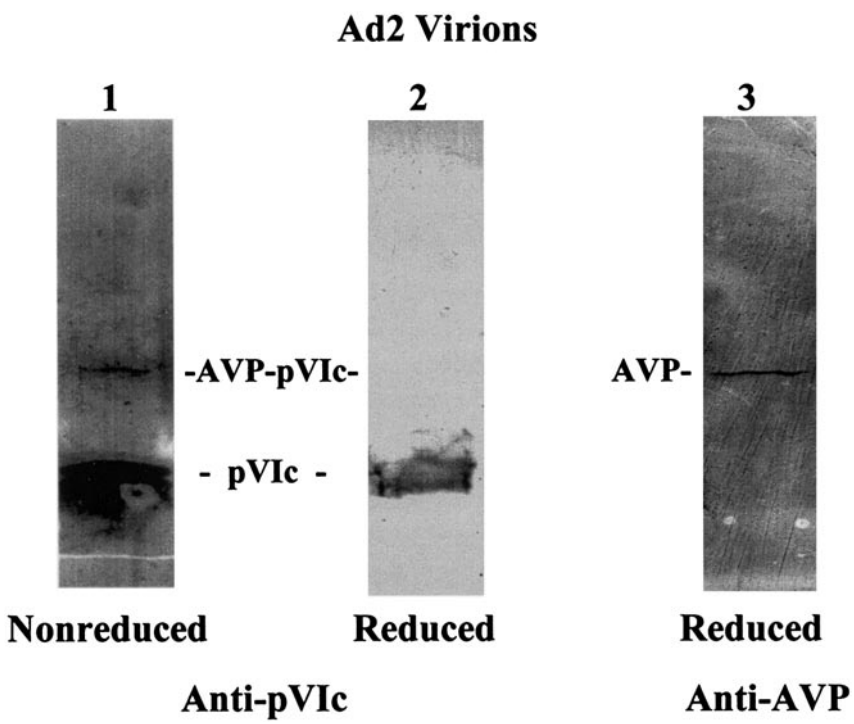

FIG. 3. Formation of a disulfide bond between pVIc and AVP in adenovirus virions. Disrupted wild-type Ad2 virions, 10^{10}, prepared by incubation at 56°C for 20 min in 10 mM HEPES (pH 8.0) and 5 mM iodoacetamide, were placed in SDS–PAGE sample buffer plus or minus 5% β-mercaptoethanol and then sonicated for 5 min prior to being put in a boiling water bath for 3 min. After SDS–PAGE, the proteins in the gels were transferred to a treated Immobilon membrane, fixed with glutaraldehyde, and immunoblotted with either anti-pVIc antibody or anti-AVP antibody, as indicated.

DISCUSSION

The experiments described here show conclusively that in the adenovirus particle, a disulfide bond is formed between AVP and pVIc. This is the first experimental proof that AVP interacts *in vivo* with one of its cofactors, all of which were discovered and characterized *in vitro* (Mangel *et al.,* 1993; Webster *et al.,* 1993). Our model for the activation of AVP in the immature virion postulates that AVP bound to the viral DNA encounters pVI and cuts out pVIc, which then binds to the enzyme that cut it out (McGrath *et al.,* 2001a). The equilibrium dissociation constant, K_d, for the reversible binding of pVIc to AVP is 4.4 μM (Baniecki *et al.,* 2001). In the presence of 12-mer ss-DNA, the K_d decreases to 0.09 μM. Apparently, the binding of DNA to AVP makes the pVIc binding site on AVP more accessible. Eventually a covalent bond is formed between AVP and pVIc. Even though pVIc is a basic protein that binds to DNA with an apparent K_d of 693 μM (McGrath *et al.,* 2001a), a conclusion from the experiments in Figs. 1 and 2 is that pVIc can bind and form a disulfide bond with AVP in the presence of Ad2 DNA.

pVIc reversibly bound to AVP can stimulate enzyme activity (Baniecki *et al.,* 2001; McGrath *et al.,* 2001b). Furthermore, the maximal rate of substrate hydrolysis by the reversible AVP–pVIc complex is equal to the rate of substrate hydrolysis of disulfide-linked AVP–pVIc complexes (McGrath and Mangel, unpublished results). Upon addition of monomeric pVIc to AVP, the maximal rate of substrate hydrolysis was achieved within 3 min; in the presence of DNA, the maximal rate of substrate hydrolysis was reached in less than 1 min.

There have been conflicting reports as to whether Cys10′ in pVIc is essential for the reversible binding of pVIc to AVP. Using mass spectrometry as an assay for binding, the pVIc mutant C10′S (GVQSLKRRRSF) was shown not to bind to AVP (Cabrita *et al.,* 1997). However, the concentrations of the C10′S mutant used, up to 40 μM, are too low to see binding. The C10′S mutant does bind to AVP and upon binding stimulates enzyme activity; the K_d for binding is quite high, greater than 400 μM (McGrath *et al.,* 2001b). Ruzindana-Umunyana *et al.* (2000) were able to titrate AVP with the C10′S mutant and concluded that formation of a disulfide bond between pVIc and AVP is not necessary for pVIc to stimulate enzyme activity. However, analysis of their titration data indicated the K_d is less than 2 μM (our inference). Although we obtained a K_d much greater than 400 μM for the binding of the C10′S mutant to AVP, in the presence of 12-mer ss-DNA, the K_d dropped to 7.6 μM (McGrath *et al.,* 2001b). Thus, it appears as if their assays contained polyanion.

Before determining by Western blot analysis (Towbin *et al.,* 1979) whether a disulfide bond between AVP and

pVIc formed *in vitro* or in the virus particle *in vivo,* certain aspects of the Western blotting procedure had to be investigated. pVIc is a small peptide (MW 1350) and although it can be fractionated as a diffuse band upon SDS–PAGE and be transferred efficiently from gel to membrane, it is not efficiently retained on the membrane support during the long incubations with antibodies and washes (Tovey and Baldo, 1989). To enhance the retention of pVIc on a membrane, the membrane was pretreated with BSA, proteins were electrophoretically transferred to the membrane after SDS–PAGE, and then the transferred proteins were cross-linked by glutaraldehyde to the BSA bound to the membrane. The results indicated an approximate 2000-fold increase in the retention of pVIc on the membrane (McGrath and Mangel, unpublished results).

This method of Western blotting was validated when it was used to demonstrate the presence of a disulfide bond in AVP–pVIc complexes formed *in vitro* (Fig. 1). The pVIc in the AVP–pVIc complexes could be clearly seen on the blot from the nonreduced gel but not on the blot from the reduced gel. The 7:1 molar ratio of pVIc to AVP used in the experiment in the absence of DNA was similar to that found in wild-type Ad2 virions (Brown *et al.,* 1996; Oosterom-Dragon and Anderson, 1983).

This new method of Western blotting was used to measure the kinetics of disulfide bond formation *in vitro* between AVP and pVIc in the absence or presence of Ad2 DNA. Previously, this had been done in the absence of DNA by an indirect method wherein enzyme activity that remained after dilution below the K_d for AVP and pVIc signified formation of a disulfide bond (McGrath *et al.,* 2001b). The results of the direct experiments in Fig. 2 confirmed that the rate of disulfide bond formation was relatively slow; the majority of AVP appeared not to have formed a disulfide bond with pVIc within 6 h. These results then reinforce the conclusion that formation of a disulfide bond between AVP and pVIc is not necessary for maximal stimulation of AVP by pVIc. Since upon addition of monomeric pVIc to AVP the maximal rate of substrate hydrolysis was achieved within 3 min (McGrath *et al.,* 2001b), the results of the direct experiment in Fig. 2 reinforce the conclusion that formation of a disulfide bond between AVP and pVIc is not necessary for the enzyme to be maximally stimulated by pVIc.

This method of Western blotting was then used to show that *in vivo,* in the virus particle, pVIc had formed a disulfide bond with AVP (Fig. 3). In the blot of the nonreduced gel, a band illuminated by anti-pVIc antibody comigrated with AVP–pVIc. That band was absent in the blot of the reduced gel. During disruption of the virus before SDS–PAGE, 5 mM iodoacetamide was present. This ensured that all free thiols would become alkylated, and thus, no new disulfide bonds could form *in vitro.*

Greber *et al.* (1996) have also fractionated virion proteins under reducing and nonreducing conditions. Their Western blot was probed with an antibody directed against AVP, whereas we used an antibody directed against pVIc. To see if pVIc (MW 1350) binds to AVP (MW 23,000) using an anti-AVP antibody, the gel would have to resolve two proteins with molecular weights of 23,000 and 24,350. This is why the authors conclude that their results are consistent with the presence of an interchain disulfide that connects AVP to pVIc. Furthermore, there are several low-molecular-weight peptides present in adenovirus virions; using an antibody directed against AVP would not differentiate between those small peptides and pVIc. The results presented here are more definitive, because an anti-pVIc antibody is used; in this case the gel has only to resolve two proteins with molecular weights of 1350 and 24,350 (Figs. 1–3).

The data in Fig. 3 are consistent with the conclusion reached via indirect evidence that there are about 70 proteinase molecules per virion (Brown *et al.,* 1996). There are 340 molecules of pVI per virion (van Oosterom and Burnett, 1985). The immunoblots in Fig. 3 indicated that in the virus apparently all the pVI had been processed; there was no detectable pVI, only pVIc. The pVIc band at the bottom of the blot in lane 1 in Fig. 3 is much more intense than the band associated with AVP–pVIc complexes. This quite possibly reflects a ratio of pVIc to AVP predicted to be 340:70 (Brown *et al.,* 1996; Oosterom-Dragon and Anderson, 1983). At the very least, it indicates that there are many more molecules of pVIc per virion than AVP.

If the formation of a disulfide bond between pVIc and AVP is not necessary for maximal stimulation of enzyme activity by pVIc, why does this bond form *in vivo,* in the virus particle? Perhaps the covalent attachment of pVIc to AVP is important to enzyme activity, but in an indirect way. Given the relatively low concentration of pVI and AVP in the virion, reversible binding of pVIc to AVP might not lead to activation of all the molecules of AVP all the time. This might prevent all the precursor proteins from being processed. By forming a covalent bond, an AVP molecule becomes irreversibly activated; i.e., pVIc cannot reversibly dissociate due to its low concentration. As to how the covalent bond is formed initially, perhaps AVP, bound to the viral DNA, encounters pVI and cleaves out pVIc, which in turn binds to the enzyme that cut it out. The pVIc would bind to the AVP bound to the DNA because the equilibrium dissociation constant of AVP for pVIc is 4.4 μM in the absence of DNA and 90 nM in the presence of DNA (Baniecki *et al.,* 2001).

MATERIALS AND METHODS

Materials

Recombinant AVP was purified as previously described (Mangel *et al.,* 1996). pVIc was purchased from Research Genetics (Huntsville, AL). BSA, Ad2 DNA, and Ellman's reagent, 5,5′-dithio-bis(2-nitrobenzoate) or DTNB

were purchased from Sigma Chemical Co. (St. Louis, MO). Glutaraldehyde was purchased from Kodak (Rochester, NY). Polyclonal antibodies elicited by pVIc or AVP were purchased from Cocalico Biologicals Inc. (Reamstown, PA). Triton X-100, SDS, BCIP/NBT Color Development Solution, and blotting grade affinity-purified goat anti-rabbit IgG (H+L) alkaline phosphatase conjugate human IgG adsorbed were purchased from Bio-Rad Laboratories (Hercules, CA). Polyacrylamide gel electrophoresis and electrophoretic transfer materials were purchased from Pharmacia LKB Technologies (Piscataway, NJ). Immobilon P transfer membrane (LeGendre, 1990) was purchased from Millipore (Boston, MA).

Protein concentration

Protein concentration was determined by the BCA protein assay (Pierce, Rockford, IL). For AVP, the concentration was also determined by absorbance using a calculated molar extinction coefficient at 280 nm of 26,510 (Gill and von Hippel, 1989). The concentration of pVIc was determined by titration of its cysteine with Ellman's reagent; results were confirmed by quantitative amino acid analysis. The titration was done by adding 10 μl of stock pVIc solution to 0.99 ml of 0.33 mM DTNB in Ellman's buffer, which contained 0.1 M NaH_2PO_4 (pH 7.3) and 1 mM EDTA. The absorbance at 412 nm was read. Moles of thiolate anion were calculated using a molar extinction coefficient for thionitrobenzoate at 412 nm of 14,150 (Riddles *et al.,* 1979).

Formation of disulfide-linked AVP–pVIc complexes

Complexes of AVP and pVIc (AVP–pVIc) were formed by incubating 7 μM AVP with 50 μM pVIc in 10 mM Tris–HCl (pH 8.0) for varying periods of time at 25°C. Complexes of AVP and pVIc were formed in the presence of Ad2 DNA by incubating 2 μM AVP with 2.5 μM pVIc in the presence of 17 μg/ml Ad2 DNA in 10 mM Tris–HCl (pH 8.0) for varying periods of time at 25°C. The reactions were diluted 15-fold with buffer prior to SDS–PAGE.

Antibodies

The anti-pVIc antibody, elicited in rabbits as a BSA conjugate, was affinity purified using a keyhole limpet hemocyanin–pVIc peptide conjugate coupled to AminoLink Gel from Pierce. The pVIc peptide conjugate was made using the Imject maleimide activated keyhole limpet hemocyanin from Pierce. Conjugation and coupling of the conjugate to AminoLink Gel were performed according to the manufacturer's instructions. Affinity-purified pVIc antibody was stored at −20°C.

Polyclonal antibodies elicited against purified AVP were raised in rabbits and the sera were diluted 1:200 in blocking buffer prior to use.

SDS–polyacrylamide gel electrophoresis and transfer of proteins to membranes

SDS–polyacrylamide electrophoresis was carried out in 8–25% gradient gels using the Pharmacia PhastSystem according to the manufacturer's instructions. Sample buffer contained 0.01 M Tris–HCl (pH 8.0), 0.001 M EDTA, 2.5% (w/v) SDS, and 0.01% (w/v) bromophenol blue; for reduced gels, 5% (v/v) β-mercaptoethanol was also present in the sample buffer. After electrophoresis, the gel was soaked for 10 min in 0.05 M HEPES (pH 7.5) containing 20% (v/v) glycerol. Protein transfer from gel to Immobilon-P membranes was done with the semidry transfer apparatus from Pharmacia using the times and currents recommended by the manufacturer.

Preparation of membranes for transfer

The membranes were prepared for transfer by incubating them for 30 min in 0.01 M TAPS (pH 8.6) containing 0.025% (w/v) BSA. Just prior to being placed under the gel, the membrane was dipped in anode transfer buffer, 0.01 M TAPS (pH 8.6) with 35% (v/v) methanol. The gel was cut away from its Mylar backing with a taut wire. The semidry electrophoretic transfer method involved the use of filter papers soaked in transfer buffers placed over and under the gel/membrane sandwich. The anode buffer was 0.01 M TAPS (pH 8.6) containing 35% (v/v) methanol. The cathode buffer was 0.01 M TAPS (pH 8.6) containing 0.1% (w/v) SDS and 20% (v/v) methanol. Transfer took place at 20 V (25 mA) for 5 Vh at 15°C. Afterward, the membrane was placed in 0.01 M TAPS (pH 8.6) containing freshly prepared 0.25% (v/v) glutaraldehyde and incubated for 15 min at room temperature. Glutaraldehyde cross-links free primary amines, i.e., the primary amine at the N-terminus of proteins and the ϵ-amino group of lysine residues. Fixation should not be done in buffers with primary amines such as Tris–HCl. After fixation, the glutaraldehyde was quenched by incubating the membrane in 0.2 M Tris–HCl (pH 7.5) for 5 min. The membrane was rinsed with distilled water and incubated in blocking buffer, 0.1 M Tris–HCl (pH 7.4) containing 0.9% (w/v) NaCl and 3% (w/v) BSA, for at least 1 h.

Antibody binding and development of the blot

For antibody binding, the membrane was rinsed with distilled water and incubated for 2–16 h with a 1:200 dilution in blocking buffer of primary antibody, either anti-AVP or anti-pVIc. This and all subsequent incubations were performed at room temperature in solutions that were gently rocked back and forth, to facilitate diffusion. The incubation was followed by four 10-min washes with 0.01 M Tris–HCl (pH 7.4) containing 0.9% (w/v) NaCl, 0.1% (w/v) BSA, 0.1% (v/v) Triton X-100, and 0.05% (w/v) SDS (TBSXS) and a 5-min wash in blocking buffer. The membrane was then incubated for 1–2 h with

the secondary antibody (goat anti-rabbit IgG–alkaline phosphatase conjugate) solution (1:1500 dilution in blocking buffer). After four more 10-min washes with TBSXS, the membrane was washed for 10 min with PBS and then developed using the NBT/BCIP methodology (Bio-Rad Laboratories). After color development, the membrane was rinsed with distilled water, incubated in 0.05 M sodium acetate (pH 5.0) containing 0.025 M EDTA for 30 min, rinsed with water, and dried.

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